In the United States Patent And Trademark Office

In re Patent Application of:
Farooqui, F. *et al.*

Serial No.: 10/032,592

Filed: October 24, 2001

For: **Efficient Synthesis of Protein-Oligonucleotide Conjugates**

Examiner: Ceperley, M.

Art Unit: 1641

Atty. Dkt.: 2065-181 (3501.143)

Declaration Pursuant to 37 C.F.R. § 1.131

Honorable Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. We, Firdous Farooqui and M. P. Reddy, are the inventors of the above-identified patent application.

2. We have been advised that the claims of the present application have been rejected as anticipated (pursuant to 35 U.S.C. § 102(e)) or as obvious (pursuant to 35 U.S.C. §§ 102(e)/103(a)) in light of U.S. Patent Publication US 2003/0045694 A1 (Chait *et al.*), taken alone or in combination with U.S. Patent No. 5,648,213 (Reddy *et al.*).

3. Prior to the August 13, 2001, filing date of Chait *et al.*, we conceived of the present invention, and reduced it to practice in the United States. As evidence thereof, we have enclosed true copies of pages of the laboratory notebook of Dr. Firdous Farooqui (**Exhibit A**), as well as a true copy of an "Invention Disclosure" (**Exhibit B**).[2] The documents comprising **Exhibits A** and **B** were prepared and completed by us, or under our direction and supervision, in the United States prior to August 13, 2001. The experiments referred to in these Exhibits were conducted and completed in the United States prior to August 13, 2001.

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[2] The dates and names of witnesses have been redacted from the documents.

A. **Exhibit A**, pages 1-2 describes the synthesis of an oligonucleotide of defined sequence, its purification, 3' amination conjugation with an anti-IL-8 antibody. **Exhibit A**, page 3 describes the purification of the antibody-oligonucleotide conjugate. **Exhibit A**, pages 4-5 describe the use of the conjugate molecule to assay IL-8.

B. **Exhibit B** describes embodiments of the present invention, including a preferred reaction scheme, and a photocopy of the results of an assay of IL-8 that was actually conducted in the United States prior to April 13, 2001, using the antibody-oligonucleotide conjugate.

4 We each hereby declare that all statements made herein of our own respective knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully Submitted,

Oct 04, 2004
Date

Firdous Farooqui
Firdous Farooqui

Oct 04, 2004
Date

M. P. Reddy
M. P. Reddy

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Exhibit A

1L8 - oligo conjugate

Oligo - NH_2 3' 5' CGC·TAC·TTC·GAC·AGC·ATC·GCC·AGT·GT3·TA-TB' 3'/NH_2 (oligo-2)

40 ods G-25 purified

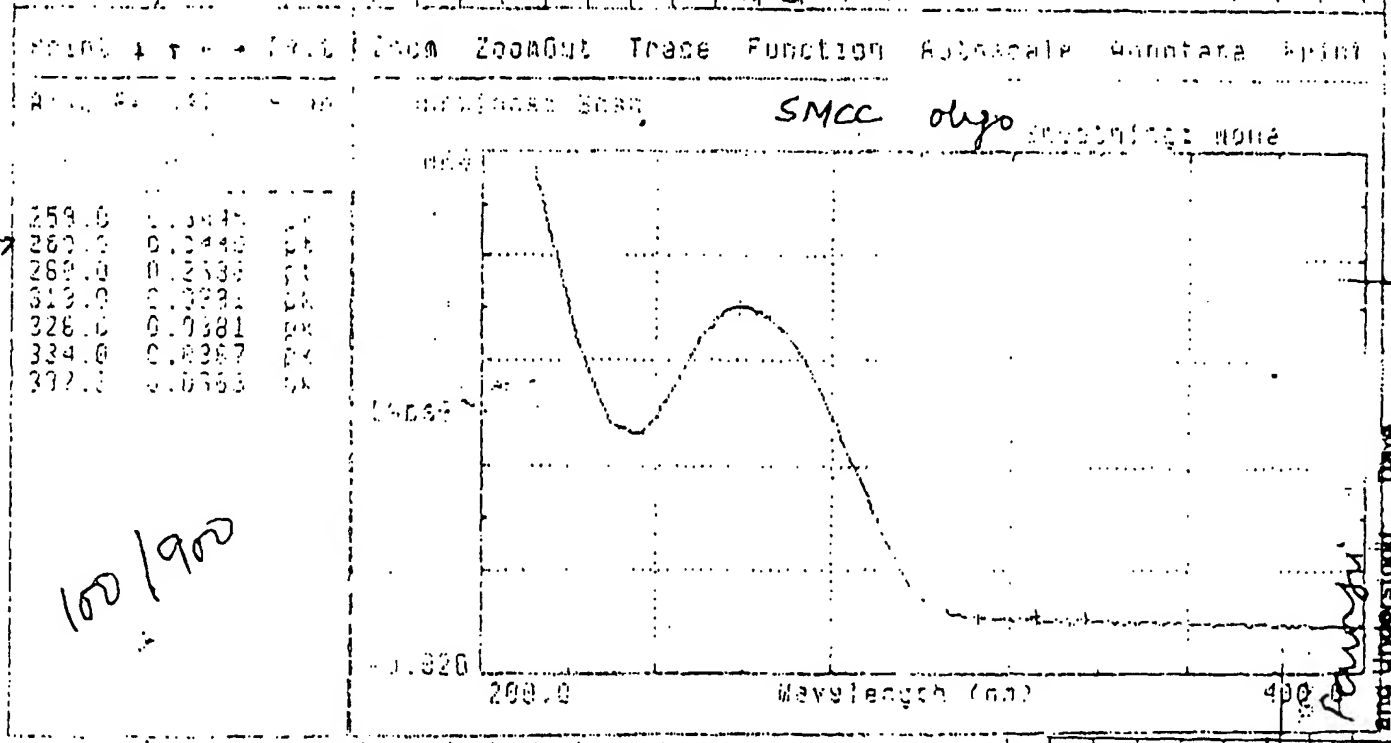
40 ods - 3'- amino oligo + 4.6 mg sulfo SMCC in 500 µl of 0.1 M HCO_3^- buffer pH 8.2 Incubate at rt for 1 hr. Purify on G-25 superfine DNA grade 1.5 x 50 cm column using H_2O.

1 min fraction collected.

fr. #	260 nm	
11	2.5	Mixed 11-14
12	2.5	
13	2.5	
14	1.5	
15	0.4976	

100 µl / 900 µl SMCC Oligo.

Total volume = 10 mL



100/900

Total = 34.45 ods

Witnessed and understood: Dang

Farooqui

α IL8 antibody R & D sample

2.5 mg dialyzed using NAP-25 with 1X PBS
Concentrated using centricon-30

IT 2 mg/mL
138 X 30 = 4140
1,00,000 4140
2.5 0.07 mg

5 mg / 1000 µl
07 mg 14 µl

α IL8 Ab in ~ 600 µl of 1X
+ (14 µl IT solution & ~20)
incubate at rt for 2h
Add.

Passed through G-25, 1X PBS 5mM EDTA

1 min fraction

fr. #	280 nm
10	0.0017
11	0.0017
12	0.1628
13	0.5002
14	0.0251
15	0.0108

mixed ~ 8.5 mL

Total volume = 19 mL SMCC oligo + Ab-1

Add 3M salt = 3.33 gms

2mM EDTA = 380 µl of 100 mM

10X PBS = 1 mL

Put 2.3 mL in centricon-30 & centricon and centrifuge at 6000 rpm for 8 hrs 22°C Flow at 6000 rpm for another 12°C 3 hrs & leave over 3 nights.

NaCl
MW = 58.44 175.32 gm 1 mL 3M

Work Performed by: F. Farooqui Date
Witnessed and Understood: Date

P-100 column of 128 Ab conjugate
from page (106-107) purified

P100 column (98-180μ) 1.5 x 25 cm column
w 0.1 M Tris 5mM EDTA pH 7.4 3 min fractions

fr #	260 nm	280 nm	260/280		base line
5	0.0411	0.0466			.03
6	0.0111	0.0205			.01
7	0.0423	0.0971			.02
8	0.2817	0.2675	1.053		.02
9	0.6926	0.6674	1.037	8-11 Mixed	.02
10	0.5025	0.4621	1.0874		.03
11	0.3188	0.2347	1.3583		.01
12	0.3750	0.2311	1.6226		.01
13	0.5144	0.3269	1.5736		.02
14	0.6649	0.3859	1.7216		.01
15	1.2437	0.7036	1.7673		.01

Mix fractions 8-11 and pass through DEAE
1 cm x 3 mL DEAE gel, wash w 10 mL 0.1 M Tris pH 8.6
5 mM EDTA then load sample 4X

	fr #		260 nm	280 nm	260/280		base line
	1	unbound sample	0.2801	0.2828	0.9964		.05
	2	wash	0.1064	0.1091			.002
	3	wash	0.0397	0.0363			
0.1 M Salt	4		0.0357	0.0260		2 mL	.006
0.1 M Salt	5		0.0569	0.0493		2 mL	.02
0.25	6	A	0.1491	0.1401	1.064	2 mL	.002
0.25	7	B	0.1262	0.1210	1.007	2 mL	.01
0.5	8	C	0.2887	0.2356	1.226	2 mL	.004
0.5	9	D	0.1943	0.1614	1.204	2 mL	.01
1	10		0.0923	0.0814	1.1339	2 mL	.01

Same sample 4 5 6 A DB 0-15
6 in PAGE

A+B mixed ran 8% PAGE
C+D mixed

IL8 assay using conjugate from page 117 IL8-Cubicle Ab conjugate

Step I 40µl of reagent + 40µl of 0.5 salt in casein + 320µl of casein buffer

	1	2	3	4
pg 117	Ab #	A	B	C	D

Add 50µl in each well, shake @ 37°C for 1hr
wash 3X 5 min each, wash buffer from 0.5M salt

Step II 148 1µl ——→ 1mL 10 ng/mL (100µl of 5M salt + 900µl of casein buffer to make 0.5M salt)
↳ 25µl ——→ 1mL 250 pg/mL
↳ 400µl ——→ 1mL 100 pg/mL
↳ 250µl ——→ 1mL 25 pg/mL

Add 50µl & shake at 37°C for 1hr.
wash 3X, 5 min each.

Step III Ab-biotin 1:100
10:1000
20:2000

1½ hr at 37°C, wash 3X 5 min each

Step IV SAv-AP
1:500
2:1000

SAv-PBXL
1:100
10:1000

} 50 min at 37°C

Substrate
1:20
10:200
40:800

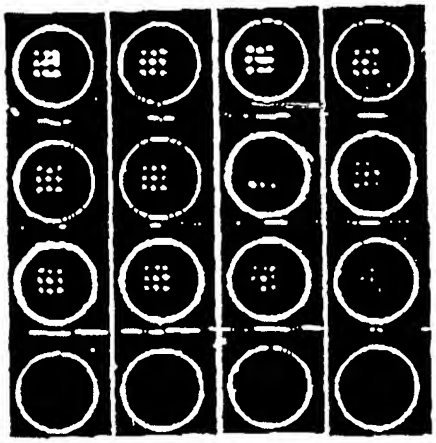
30 min at rt No shaking

Wash & take picture.

EXAMINER

ELF

IL8



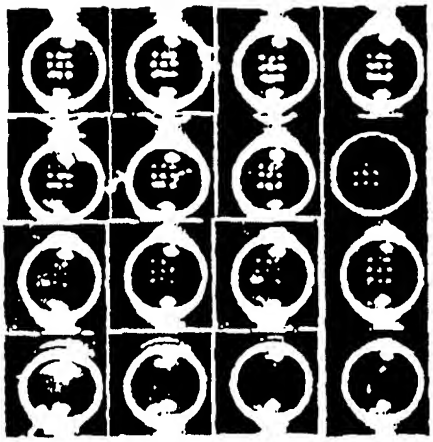
250 pg/ml

100 pg/ml

25 pg/ml

0 pg/ml

PBxl



250 pg/ml

100 pg/ml

25 pg/ml

0 pg/ml

Work Performed by: F. Farooqui Date

Witnessed and Understood: Date

Mixed fractions (A+B from page 117 IL8 - whole Ab conjugate
2C+D
run on gel 10% denaturing Page by Dean.
Conclusion: IL8 - dip conjugate perform well in assay

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Exhibit B

INVENTION DISCLOSURE

1. **Title of Invention**: Efficient synthesis of protein-oligonucleotide conjugates.

2. **Product line in which invention will be used:** Immediate use in Protein array or A^2 plate project. Potential use in bead based flow project. This new chemistry may be used to manufacture protein-oligonucleotide conjugates in high yields.

3. **Summary of the Invention**: Oligonucleotide-antibody conjugates are useful reagents in immunoassays[1]. The covalent attachment of an oligonucleotide molety to antibody provides a specific recognition site for a complementary nucleic acid and can subsequently be used as a molecular handle for selective immobilization and construction purposes. In a multiplexed immunoassay approach, oligonucleotide arrays printed on A^2 plate serve as a universal capture reagent. The complimentary oligonucleotides are conjugated to the corresponding antibodies and the assay is performed. The antibody-conjugated probe prepared by the method disclosed here gave high sensitivity.

Synthesis of oligonucleotide-antibody conjugate is accomplished in four steps: 1) Synthesis of 3'-amino oligonucleotide 2) 3'-amino oligonucleotide is activated with a hetero bifunctional linker, sulfo SMCC 3) antibodies are thiolated using iminothiolane (Traut's reagent) 4)The activated oligonucleotide and thiolated antibodies are then mixed to facilitate coupling of oligonucleotide to antibody (see Figure 1). Antibody-oligonucleotide conjugates are characterized by PAGE and tested for their biological activity by performing immunoassay (see Figure 2). This approach is simpler or higher yielding as compared to the procedures known in literature: thiol group is introduced at the 5' or 3' end of oligonucleotide

Inventors Name:	Date
Firdous Farooqui F. Farooqui	
M. Parameswara Reddy M. Parameswara Reddy	
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antibody was purified on G-25 column, 1.5 cm x 50 cm, using 1 x PBS, 5 mM EDTA as an eluent.

4. DNA oligonucleotide-Ab conjugation: Activated antibody was mixed with sulfo SMCC oligonucleotide, the final conc. was 1 x PBS, 3 M NaCl, 2 mM EDTA. The resulting mixture was concentrated using centricon-3 for 3 hours and left to react at 12°C for 3 nights.

5. Purification of Oligonucleotide antibody conjugates: The Antibody-oligonucleotide conjugate was purified on size exclusion P-100 column, 1.5 cm x 20 cm, using 0.1 M tris buffer pH 7.2, 5mM EDTA as an eluent.The first band collected which had 260/280 ratio of 1.0- 1.2 were mixed and purified on DEAE column with salt gradient from 0.5 M NaCl to 1 M NaCl. The yield of the conjugate was 25-35%. The conjugate was characterized on 8% PAGE and stained with stains all.

6. Immunoassay Protocol: The complementary oligonucleotide was printed on A^2 plate 3 x 3 array. The 140 ng of Antibody-oligonucleotide in casein buffer with 0.5 M salt was added to the well and incubated for 1hr at 37°C. Wash the plate 3x, added antigen in different conc. ranging from 1000 to 1 pg/ml in 50 ul of volume, wash 3x, added secondary antibody conjugate with biotin react for 1 hour at 37°C, wash 3x, and added SAv-PBXsl react for 1hour wash 3x and imaged the plate under CCD camera. The sensitivity of the assay was found to be 1 pg/ml with IL8 antigens (see Figure 2).

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Figure 1

Synthesis of Oligonucleotide-Antibody Conjugate

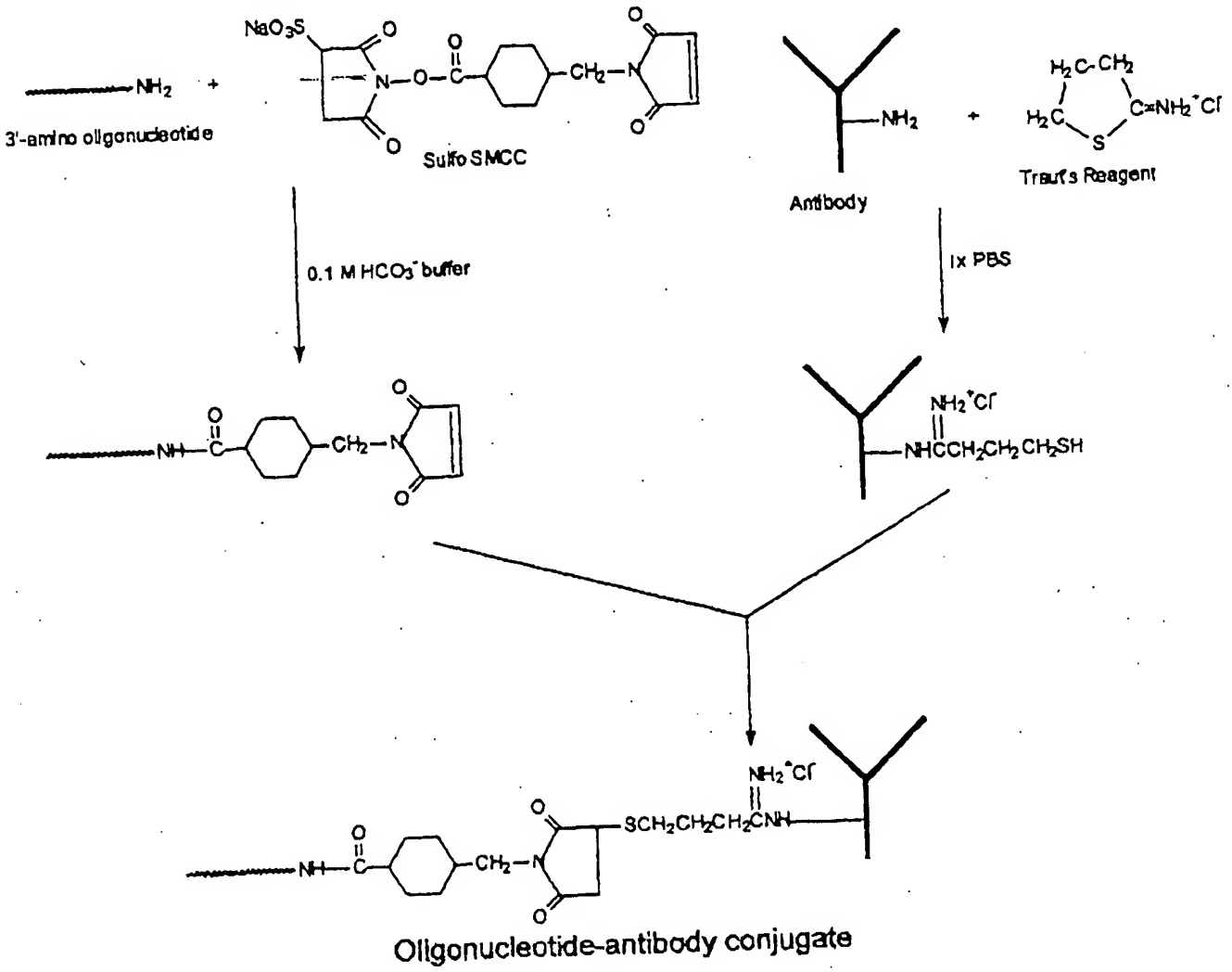

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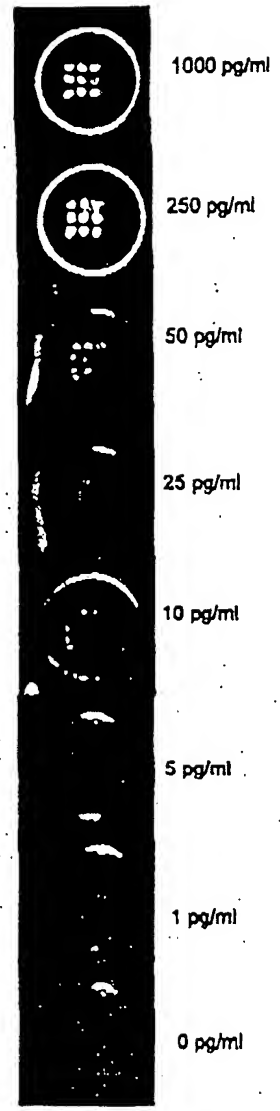
Figure 2

IL 8 assay on A^2 Plate



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Witnessed and Understood	Date
Witnessed and Understood	Date

Claims: Synthesis of oligonucleotide-antibody conjugates where oligonucleotide is activated with a hetero bifunctional linker, antibody is thiolated and mixed to form a conjugate.

References:

1. M. P. Reddy, J. C. Sternberg; US patent # 5,648,213.
2. Yarmush M. L. et at; Bioconjugate chemistry 8 (6) 935-940, 1997.
3. Hendrickson R. E. et al Nucleic Acid Research 23 (3), 522-529, 1994.
4. DNA Probes, George H. Keller, Mark M. Manak, MacMillan Publishers Ltd, 1989.

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